



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07K 1/14, 14/475, 14/48, C12N 1/20, 15/00, 15/18		A1	(11) International Publication Number: WO 95/30686 (43) International Publication Date: 16 November 1995 (16.11.95)
(21) International Application Number: PCT/US95/05423 (22) International Filing Date: 2 May 1995 (02.05.95) (30) Priority Data: 08/240,122 9 May 1994 (09.05.94) US 08/266,080 27 June 1994 (27.06.94) US (71) Applicant: THE SYNTEX-SYNERGEN NEUROSCIENCE JOINT VENTURE [US/US]; 3401 Hillview Avenue, P.O. Box 10850, Palo Alto, CA 94303 (US). (72) Inventors: LILE, Jack; 947 Casitas Vista Road, Ventura, CA 93001 (US). KOHNO, Tadahiko; 1557 Hays Court, Louisville, CO 80027 (US). BONAM, Duane; 4 Morsecroft Lane, Amesbury, MA 01913 (US). ROSENDAHL, Mary, S.; 310 Fairplay, Broomfield, CO 80020 (US). (74) Agents: SWANSON, Barry, J. et al.; Swanson & Bratschun, L.L.C., Suite 200, 8400 E. Prentice Avenue, Englewood, CO 80111 (US).		(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LR, LT, LU, LV, MD, MG, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ, UG). Published <i>With international search report.</i>	
(54) Title: PRODUCTION OF BIOLOGICALLY ACTIVE RECOMBINANT NEUROTROPHIC PROTEIN			
(57) Abstract A process for the production of biologically active recombinant neurotrophic factor from the NGF/BDNF family is described. The process is comprised of: a) constructing a synthetic neurotrophic factor gene suitable for expression in a bacterial expression system; b) the synthetic neurotrophic factor gene is expressed in a bacterial expression system; c) the neurotrophic factor is solubilized and sulfonylated; d) sulfonylated neurotrophic factor is allowed to refold in the presence of polyethylene glycol and urea; and e) biologically active neurotrophic factor is isolated and purified.			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

-1-

PRODUCTION OF BIOLOGICALLY ACTIVE RECOMBINANT
NEUROTROPHIC PROTEIN

5 FIELD OF THE INVENTION

 This invention relates to processes for the
production of recombinant nerve growth factors from the
NGF/BDNF family. Specifically, the present invention
describes a method for producing biologically active
10 recombinant NGF, BDNF, NT3 and NT4.

BACKGROUND OF THE INVENTION

 Neurotrophic factors are natural proteins, found
in the nervous system or in non-nerve tissues
15 innervated by the nervous system, whose function is to
promote the survival and maintain the phenotypic
differentiation of nerve and/or glial cells (Varon and
Bunge (1978) Ann. Rev. Neurosc. 1:327; Thoenen and
Edgar (1985) Science 229:238). In vivo studies have
20 shown that a variety of endogenous and exogenous
neurotrophic factors exhibit a trophic effect on
neuronal cells after ischemic, hypoxic, or other
disease-induced damage. Examples of specific
neurotrophic factors include basic fibroblast growth
25 factor (bFGF), acidic fibroblast growth factor (aFGF),
nerve growth factor (NGF), ciliary neurotrophic factor
(CNTF), brain derived neurotrophic factor (BDNF),
neurotrophin 3 (NT3), neurotrophin 4 (NT4), and the
insulin-like growth factors I and II (IGF-I, IGF-II).

30 Some neurotrophic factors, such as bFGF and CNTF,
are thought to have broad trophic effects, promoting
survival or providing a maintenance function for many
different types of neuronal cells. Other neurotrophic
factors have a narrower, more specific trophic effect
35 and promote survival of fewer types of cells. For
example, in the peripheral nervous system NGF promotes
neuronal survival and axonal extension of certain
specific neuronal cells types such as sensory and
sympathetic neurons (Ebendal et al. (1984) Cellular and

-2-

Molecular Biology of Neuronal Development, Ch. 15, ed. Black, I.B.). However, in the central nervous system (CNS), NGF also supports the survival of cholinergic neurons in the basal forebrain complex (Whittemore et al. (1987) Brain Res. Rev. 12:439-464).

BDNF, a basic protein of molecular weight 12,300, supports some sensory neurons that do not respond to NGF (Barde et al. (1982) EMBO J. 1:549-553 and Hofer and Barde (1988) Nature 331:261-262). Neurotrophin 3 (NT3) supports survival of dorsal root ganglion neurons and proprioceptive neurons in the trigeminal mesencephalic nucleus. CNTF, a protein of about molecular weight 23,000, supports ciliary ganglion neurons in the parasympathetic nervous system, sympathetic neurons, dorsal root ganglion neurons in the sensory nervous system, and motor neurons in the CNS (Kandel et al. (1991) Principles of Neural Science, 3rd Ed., Elsevier Science Publishing Co., Inc., NY).

Some neurotrophic factors constitute a family of neurotrophic factors characterized by about 50% amino acid homology. One such family is the NGF/BDNF family, which includes BDNF, NGF, NT3 and NT4 (Hohn et al. WO 91/03569; U.S. Patent Application Serial No. 07/680,681). Both NGF and BDNF are apparently synthesized as larger precursor forms which are then processed, by proteolytic cleavages, to produce the mature neurotrophic factor (Edwards et al. (1986) Nature 319:784; Leibrock et al. (1989) Nature 319:149). There is a significant similarity in amino acid sequences between mature NGFs and mature BDNF, including the relative position of all six cysteine amino acid residues, which is identical in mature NGFs and BDNF from all species examined (Leibrock et al. (1989) supra). See Figure 2, comparing and emphasizing the similarities of human forms of BDNF (SEQ ID NO:3) and NGF (SEQ ID NO:4). This suggests that the three-dimensional structures of the mature proteins, as

-3-

determined by the location of the disulfide bonds, are similar. The mature NGFs and BDNF proteins also share a basic isoelectric point (pI).

NGF is a neurotrophic factor at least for
5 cholinergic neurons in the basal forebrain (Hefti and Will (1987) J. Neural Transm. [Suppl] (AUSTRIA) 24:309). The functional inactivation and degeneration of the basal forebrain cholinergic neurons responsive to NGF in the course of Alzheimer's disease is thought
10 to be the proximate cause of the cognitive and memory deficits associated with that disease (Hefti and Will (1987) supra). NGF has been shown to prevent the degeneration and restore the function of basal forebrain cholinergic neurons in animal models related
15 to Alzheimer's disease, and on this basis has been proposed as a treatment to prevent the degeneration and restore the function of these neurons in Alzheimer's disease (Williams et al. (1986) Proc. Natl. Acad. Sci. USA 83:9231; Hefti (1986) J. Neuroscience 6:2155;
20 Kromer (1987) Science 235:214; Fischer et al. (1987) Nature 329:65).

BDNF is a neurotrophic factor for sensory neurons in the peripheral nervous system (Barde (1989) Neuron 2:1525). On this basis, BDNF may prove useful for the
25 treatment of the loss of sensation associated with damage to sensory nerve cells that occurs in various peripheral neuropathies (Schaumburg et al. (1983) in Disorders of Peripheral Nerves, F. A. Davis Co., Philadelphia, PA).

30 In order for a particular neurotrophic factor to be potentially useful in treating nerve damage, it must be available in sufficient quantity to be used as a pharmaceutical treatment. Also, since neurotrophic factors are proteins, it is desirable to administer to
35 human patients only the human form of the protein, to avoid an immunological response to a foreign protein. Since neurotrophic factors are typically present in

-4-

vanishingly small amounts in tissues (e.g., Hofer and Barde (1988) Nature 331:261; Lin et al. (1989) Science 246:1023) and since human tissues are not readily available for extraction, it would be inconvenient to
5 prepare pharmaceutical quantities of human neurotrophic factors directly from human tissues. As an alternative, it is desirable to use the isolated human gene for neurotrophic factor in a recombinant expression system to produce potentially unlimited
10 amounts of the human protein.

Mature, biologically-active neurotrophic factors can be produced when human or animal neurotrophic factor genes are expressed in eukaryotic cell expression systems (e.g., Edwards et al., (1988) Molec.
15 Cell. Biol. 8:2456). In such systems, the full-length neurotrophic factor precursor is first synthesized and then proteolytically processed to produce mature neurotrophic factor which is correctly folded 3-dimensionally and is fully biologically active.
20 However, eukaryotic cell expression systems often produce relatively low yields of protein per gram of cells and are relatively expensive to use in manufacturing.

In contrast, expression systems that use
25 prokaryotic cells, such as bacteria, generally yield relatively large amounts of expressed protein per gram of cells and are relatively inexpensive to use in manufacturing. However, obtaining biologically active bacterially-expressed neurotrophic factor has been a
30 major hurdle in this field. Bacteria are not able to correctly process precursor proteins, such as the precursor protein for NGF, by making appropriate proteolytic cleavages in order to produce the correct smaller mature protein. Therefore, to produce mature
35 neurotrophic factor in bacteria, it is necessary to express only that portion of the DNA sequence encoding the mature protein and not that for the larger

-5-

precursor form. When this was done in E. coli, relatively large amounts of the mature human NGF protein were produced (see, e.g., Iwai et al. (1986) Chem. Pharm. Bull. 34:4724; Dicou et al. (1989) J. Neurosci. Res. 22:13; European Patent Application 121,338). Unfortunately, the bacterially-expressed protein had no apparent biological activity.

Bacterial production of recombinant mammalian proteins often result in biologically inactive proteins forming inclusion bodies. This necessitates separating the inclusion bodies from other cell components, and solubilizing the inclusion bodies to unfold the protein (Spalding (1991) Biotechnology 9:229). The likely reason for this lack of biological activity is that the mature protein is unable to assume spontaneously the correct 3-dimensional structure and form the correct intramolecular disulfide bonding pattern required for full biological activity. Processing includes the separation and solubilization of the inclusion bodies, unfolding the protein, then refolding the protein into the correct biologically active tertiary structure. However, during refolding, the protein may reaggregate, reducing the yield of active protein and further complicating the purification process (Spalding (1991) supra).

Protocols for unfolding and refolding NGF have been described (e.g., European Patent Application 336,324; U.S. Patent Nos. 4,511,503 and 4,620,948). However, these protocols have serious deficiencies. Many protocols use exposure of NGF to high pH to break incorrectly formed disulfide bonds followed by exposure to lower pH to allow formation of correct intramolecular disulfide bonds. The exposure of NGF to high pH is known to result in extensive modification of the protein, including elimination of amine side chains in glutamine and asparagine (of which there are 7 in mature human NGF), and extensive chemical alteration of

-6-

asparagine-glycine, asparagine-serine, and asparagine-threonine adjacent pairs (of which there are 2 in mature human NGF). In addition to these chemical modifications, the refolding procedure appeared to restore only approximately one-tenth of the biological activity of NGF. Although numerous protocols for refolding and renaturing proteins that do not involve harsh conditions exist, no such procedure has been applied successfully to NGF. For a general review of refolding procedures, see Kohno (1990) *Methods Enzymol.* 185:187.

Various methods have been used to improve recovery of biologically active proteins produced in a bacterial expression system. One method for cleaving incorrectly formed disulfide bonds is the use of S-sulfonated proteins obtained by sulfitolysis (U.S. Patent No. 4,421,685; Gonzalez and Damodaran (1990) *J. Agric. Food Chem.* 38:149; European Patent Application 361,830). The addition of sulfite to a protein initially cleaves the disulfide bonds exposed to the solution, resulting in the formation of one S-SO₃⁻ derivative and one free SH group for each disulfide bond cleaved. In the presence of an oxidizing agent, the free SH groups are oxidized back to disulfide, which is again cleaved by the sulfite present in the system. The reaction cycle repeats itself until all the disulfide bonds and the sulfhydryl groups in the protein are converted to cys-SO₃⁻. Generally, this allows most proteins to be fully solubilized (European Patent Application 361,830).

Another method to improve the recovery of biologically active protein from bacterial expression systems includes the use of polyethylene glycol (PEG) in the refolding mixture. It has been proposed that the addition of PEG prevents protein aggregation resulting from the association of hydrophobic intermediates in the refolding pathway. Cleland *et al.* (1990) *Biotechnology* 8:1274 and (1992) *J. Biol. Chem.*

-7-

267:13327, reported improved recovery of biologically active bovine carbonic anhydrase B (CAB) with the addition of PEG during the refolding process. The concentration of PEG required to achieve an increase in the recovery of active protein was twice the total protein concentration, and required PEG with molecular weights of 1000-8000 (Cleland et al. (1992) supra).

A bacterial expression system for producing NGF is disclosed in Canadian Patent No. 1,220,736 and U.S. Patent No. 5,169,762. However, no procedures for refolding the expressed protein are presented. A procedure for producing large quantities of biologically active recombinant NGF suitable for pharmaceutical use is described in U.S. Patent Application Serial No. 08/071,912 filed July 6, 1993 by Collins et al., entitled: Production of Biologically Active, Recombinant Members of the NGF/BDNF Family of Neurotrophic Proteins. The protein is exposed to a denaturant, such as guanidine hydrochloride or urea, and sufficient reducing agent, such as β -mercaptoethanol, dithiothreitol, or cysteine, to denature the protein, disrupt noncovalent interactions, and reduce disulfide bonds. The free thiols present in the reduced protein are then oxidized, and the protein allowed to form the correct disulfide bonds. The refolding mixture preferably contained up to 25% PEG 200 or 300.

While the procedure described in U.S. Patent Application Serial No. 08/087,912 achieves improved yields of biologically active NGF, the need remains for more efficient means for refolding NGF. The bacterial production of recombinant proteins results in biologically inactive proteins found as inclusion bodies within the bacterial cell. There is a need for improved processing methods for separating the inclusion bodies from other cell components and solubilizing the inclusion bodies to unfold the

-8-

protein. Further, there is a need for improved methods for breaking incorrectly formed disulfide bonds and refolding the protein into the correct tertiary structure required for maximum yield of fully active protein while decreasing chemical modification of the protein.

The present disclosure presents an extended and improved method for producing bacterially-expressed biologically active members of the NGF/BDNF family of neurotrophic factors, including the first use of the process of sulfitolysis to solubilize and chemically modify a neurotrophic factor.

BRIEF SUMMARY OF THE INVENTION

The present invention discloses a process for the production of mature proteins from the NGF/BDNF family in a fully biologically active form suitable for therapeutic use comprising:

- a) expressing a gene coding for the neurotrophic factor in a bacterial expression system wherein said neurotrophic factor protein is produced;
- b) solubilizing said neurotrophic factor in urea;
- c) sulfonylating said neurotrophic factor;
- d) isolating and purifying the sulfonylated neurotrophic factor;
- e) allowing the sulfonylated neurotrophic factor to refold to give the biologically active neurotrophic factor; and
- f) purifying the biologically active neurotrophic factor.

Sulfonylated neurotrophic factor is purified by anion exchange chromatography and refolded in the presence of 20% polyethylene glycol 300 (PEG 300). Refolded neurotrophic factor is purified by cation exchange chromatography.

It is to be understood that both the foregoing general description and the following detailed

-9-

description are exemplary and explanatory only, and are not restrictive of the invention, as claimed. The accompanying drawings, which are incorporated in and constitute a part of the specification, illustrate various embodiments of the invention and, together with the description, serve to explain the principles of the invention.

BRIEF DESCRIPTION OF THE FIGURES

10 FIG. 1 compares the nucleic acid sequence of human BDNF (SEQ ID NO:1) and NGF (SEQ ID NO:2). Gaps, indicated by dashes, correspond to the location of gaps used to align the amino acid sequences.

15 FIG. 2 compares the amino acid sequences of human BDNF (SEQ ID NO:3) and NGF (SEQ ID NO:4). The inferred sequences of the mature proteins are in bold. Gaps, indicated by dashes were placed in the sequences to increase alignment. The six cysteins found in BDNF and
20 NGF are found in the same locations and are bracketed.

 FIG. 3 shows the synthetic NGF sequence (SEQ ID NO:5) inserted into E. coli and expressed as the mature NGF protein.

25

 FIG. 4 shows the sequences of Mut1 (SEQ ID NO:6), Mut2 (SEQ ID NO:7), and Mut3 (SEQ ID NO:8) oligonucleotides used to correct the NGF sequence.

30 FIG. 5 shows the Syn NGF 5P oligonucleotide sequence used for making enhanced expression of NGF (SEQ ID NO:9).

35 FIG. 6 shows the TP NGF (SEQ ID NO:10) and REP NGF (SEQ ID NO:11) oligonucleotide sequences used for making the TP (TNF binding protein) NGF REP construct.

-10-

FIG. 7 shows the TP Δ53 nucleic acid sequence (SEQ ID NO:12).

FIG. 8 shows the oligonucleotide sequence used for making the TP NGF(2start-)REP construct (SEQ ID NO:13).

FIG. 9 shows a process flow diagram for the process of this invention.

10 DESCRIPTION OF THE PREFERRED EMBODIMENTS

Reference will now be made in detail to the presently preferred embodiments of the invention which, together with the following examples, serve to explain the principles of the invention.

15 The present invention is an extended and improved method for producing bacterially-expressed biologically active neurotrophic factors from the NGF/BDNF family from that disclosed in the earlier application U.S. Patent Application Serial No. 08/087,912, specifically incorporated herein by reference.

20 The production method of this invention for obtaining the fully biologically active mature human recombinant neurotrophic factor from the NGF/BDNF family is comprised of:

- 25 a) expressing neurotrophic factor in a bacterial expression system;
- b) solubilizing and sulfonylating neurotrophic factor;
- 30 c) refolding sulfonylated neurotrophic factor such that the correct tertiary structure necessary for full biological activity is obtained; and
- d) purifying the fully biologically active neurotrophic factor.

35 The present invention relates to an improved method for the efficient production of recombinant neurotrophic factors in the nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) family.

-11-

The invention herein described is a process of producing a "family" of neurotrophic growth factors in a pure and biologically active form suitable for therapeutic use. NGF is a member of a family of structurally related neurotrophic proteins which are likely to differ in their physiological role in the organism, each member affecting a different set of responsive neurons. Known members of the NGF family include NGF, BDNF, NT3 and NT4. Each of these members have significant homology and identical number of cysteine residues and location. The present invention encompasses recombinant proteins that code for proteins which are not identical to human NGF or BDNF but are clearly related to NGF or BDNF with respect to possible defining characteristics of the family. Such characteristics may include one or more of the following: neurotrophic activity in an appropriate bioassay; significant homology in amino acid sequence including both amino acid identities and conservative substitutions; conserved location of cysteine residues in the amino acid sequence; hydrophobic signal sequences for secretion of the protein; signal sequences for proteolytic processing to a mature form; and/or basic isoelectric point of the processed protein.

As used in the disclosure, the term "biological activity" when applied to NGF means proteins having the biological activity of NGF, that is for example, the ability to promote the survival of chick embryo sympathetic chain and dorsal root ganglia neurons in the bioassay described in Example 3. For other members of the NGF/BDNF family, "biological activity" means neurotrophic activity in the appropriate bioassay.

This invention encompasses the production of neurotrophic proteins of any origin which are biologically equivalent to the neurotrophic proteins of the NGF/BDNF family. In the preferred embodiment, this

-12-

invention encompasses mature human neurotrophic proteins. Throughout this specification, any reference to a neurotrophic protein should be construed to refer to the proteins identified and described herein as members of the NGF/BDNF family of neurotrophic proteins.

By "biologically equivalent" used throughout the specification and claims, we mean compositions of the present invention which are capable of promoting the survival and maintaining the phenotypic differentiation of nerve or glial cells, but not necessarily to the same degree as the native neurotrophic proteins described herein. Biologically equivalent compositions include fragments of proteins exhibiting NGF/BDNF family-like neurotrophic activity. Further encompassed by the present invention are the amino acid sequences shown in Figure 2 and those substantially homologous, with 1, 2, 3, or 4 amino acid residue changes or deletions which do not substantially alter neurotrophic activity. This invention further includes chemically modified sequences substantially homologous to those shown in Figure 2, for example, by addition of polyethylene glycol.

By "substantially homologous" as used throughout the ensuing specification and claims, it is meant a degree of homology to the native neurotrophic proteins in excess of that displayed by any previously reported neurotrophic proteins. Preferably, the degree of homology is in excess of 70%, most preferably in excess of 80%, and even more preferably in excess of 90%, 95%, or 99%. A particularly preferred group of neurotrophic proteins are in excess of 95% homologous with the native proteins. The percentage of homology as described herein is calculated as the percentage of amino acid residues found in the smaller of the two sequences which align with identical amino acid residues in the sequence being compared when four gaps

-13-

in a length of 100 amino acids may be introduced to assist in that alignment as set forth by Dayhoff, in Atlas of Protein Sequence and Structure Vol. 5, p. 124 (1972), National Biochemical Research Foundation, Washington, D.C., specifically incorporated herein by reference. Also included as substantially homologous are those neurotrophic proteins which may be isolated by virtue of cross-reactivity with antibodies to the described protein or whose genes may be isolated through hybridization with the gene or with segments of the described protein.

Members of the NGF/BDNF family of neurotrophic factors are naturally produced as larger precursor forms which are then processed by proteolytic cleavages to produce the "mature" protein (Edwards et al. (1986) supra; Leibrock et al. (1989) supra). Because bacterial expression systems are unable to correctly process the precursor form of the protein, only that portion of the DNA sequence coding for the mature protein is expressed in a bacterial expression system.

In one embodiment of the present invention, a synthetic NGF DNA sequence is constructed which is optimized for production in an E. coli expression system. The synthetic NGF gene may be constructed with a DNA sequence coding for human or animal NGF. The synthetic NGF gene is cloned into a vector capable of being transferred into and replicated in the host cell, such vector containing operational elements needed to express the DNA sequence. The construction of a preferred synthetic NGF gene and cloning into a vector suitable for transfer into an E. coli expression system is described in Example 1. This invention encompasses the use of a synthetic neurotropic factor gene from the NGF/BDNF family, as well as a synthetic gene with substantial homology to a gene from the NGF/BDNF family.

A natural or synthetic DNA sequence may be used to

-14-

direct production of NGF. The synthetic NGF gene described in Example 1, shown in Figure 3 (SEQ ID NO:5), was specifically designed to direct expression of the mature human NGF protein in a bacterial expression system. Codons for certain amino acids were optimized for expression in E. coli as well as to create restriction sites to facilitate subsequent cloning steps. The general expression method comprised:

1. preparation of a DNA sequence capable of directing E. coli to produce mature human NGF;
2. cloning the DNA sequence into a vector capable of being transferred into and replicated in E. coli, such vector containing operational elements needed to express the NGF sequence;
3. transferring the vector containing the synthetic DNA sequence and operational elements into E. coli host cells; and
4. culturing the E. coli host cells under conditions for amplification of the vector and expression of NGF.

The host cells are cultured under conditions appropriate for the expression of NGF. These conditions are generally specific for the host cell, and are readily determined by one of ordinary skill in the art in light of the published literature regarding the growth conditions for such cells and the teachings contained herein. For example, Bergey's Manual of Determinative Bacteriology, 8th Ed., Williams & Wilkins Company, Baltimore, Maryland, which is specifically incorporated herein by reference, contains information on culturing bacteria. In the preferred embodiment of the present invention, NGF is produced in an E. coli expression system. The present invention encompasses the use of this production method to produce any neurotrophic factor from the NGF/BDNF family.

A method for the production of recombinant members

-15-

of the human NGF/BDNF family of neurotrophic proteins in biologically active forms is described in U.S. Patent Application Serial No. 08/087,912 (Collins et al.). Collins et al. disclose a method for refolding and renaturing recombinant human mature members of the NGF/BDNF family of neurotrophic proteins. Any intramolecular or intermolecular disulfide bonds and/or any noncovalent interactions which have occurred involving mature neurotrophic protein produced in a microorganism are first disrupted. In order to do this, the protein is exposed to sufficient denaturant such as guanidine hydrochloride or urea, and sufficient reducing agent such as β -mercaptoethanol, dithiothreitol, or cysteine, to denature the protein, disrupt noncovalent interactions, and reduce disulfide bonds. After the mature neurotrophic protein is denatured and reduced, the free thiols present in the reduced protein are oxidized by addition of a large excess of disulfide-containing reagent, such as glutathione or cystine. This reaction produces mixed disulfide bonds in which each cysteine residue in the mature neurotrophic protein forms a disulfide bond with the monomeric form of the oxidizing agent. The denaturant and oxidizing agent are then diluted to a definite concentration and a thiol-containing reagent such as cysteine is added to catalyze disulfide interchange. This creates an environment in which the denaturant concentration is sufficiently reduced to allow the neurotrophic protein to assume various 3-dimensional configurations and in which the oxidation/reduction potential is adjusted to allow the formation and breaking of disulfide bonds. It is assumed that a significant proportion of the neurotrophic protein will form the correct intramolecular disulfide bonding pattern, and therefore, the correct 3-dimensional structure and attain biological activity. Collins et al. further

-16-

disclose the use of up to 25% polyethylene glycol (PEG) 200, 300, or 1000 added to the final refolding mixture. In the presence of PEG, a greater than 30% increase is obtained in the amount of properly-refolded
5 biologically-active NGF. The Collins et al. method represents an improvement over the harsh conditions of the prior art methods, achieving protein with up to 50% of biological activity.

The production method of the present invention
10 represents an extension and improvement over the method described by Collins et al. in several ways. The process of sulfitolysis is used to solubilize the insoluble protein produced in E. coli. Sulfitolysis imparts several important advantages to the NGF
15 purification process over all prior art methods. Sodium sulfite is a strong reductant and functions at least as well as 2-mercaptoethanol or dithiothreitol in solubilizing NGF from the washed solids. NGF fully reduced in the presence of urea does not resolve as a
20 clear peak on chromatographic resins. In contrast, sulfonylated-NGF shows a marked improvement in resolution on ion-exchange resins. Further, sulfitolysis imparts a negative charge to the protein for each cysteine which has been modified. Each
25 monomer of NGF contains six cysteine residues, and thus the fully sulfonylated monomeric form contains an additional six negative charges. This represents several advantages: 1) the increase in the total charge on each monomer increases its hydrophilicity and
30 solubility; 2) the additional negative charges lower the effective isoelectric point of the protein. This allows the use of anion exchange chromatography at a lower pH than is possible with the fully reduced form of the protein. Additionally, this allows for the
35 purification of a urea-solubilized form of the protein with an apparent pI of about 7.5, followed by purification of the soluble refolded form of the

-17-

protein with a theoretical pI of about 10.4. The ability to purify two forms of the same protein which exhibit different apparent isoelectric points provided the basis for the high level of separation of NGF away from contaminating E. coli proteins.

Example 2 describes the refolding and purification process of the present invention after expression of NGF in the E. coli expression system. The E. coli host cells are lysed, and the fraction containing NGF isolated as the "NGF washed solids suspension".

NGF is solubilized and sulfonylated in the presence of urea and sulfite.

Sulfonylated NGF may be captured and purified by anion exchange chromatography by several different schemes. In one embodiment, described in detail in Example 2, sulfonylated NGF is diluted with Buffer A (8 M urea, 20 mM Tris-HCl, pH 9.0), applied to an anion exchange column, and eluted with Buffer B (8 M urea, 36 mM MES, pH 6.0). In another embodiment, sulfonylated NGF was eluted from the column with a linear gradient from Buffer A to Buffer B. In a preferred embodiment of the invention, sulfonylated NGF was diafiltered against Buffer A in an ultrafiltration cartridge, applied to an anion exchange column, and eluted with a linear gradient from Buffer A to Buffer B. In another preferred embodiment, sulfonylated NGF was concentrated and diafiltered in an ultrafiltration membrane in a stirred cell, applied to an anion exchange column, and eluted as above.

Purified sulfonylated NGF may be refolded by several methods, as described in Example 2. Urea and PEG were added to a carboy and the solution cooled. NGF was added, the pH adjusted, and solid cysteine added. The carboy was then stored at 10°C for 4 days.

Properly refolded NGF was captured by cation exchange chromatography. Refolded NGF was recovered as a single peak of protein which contained several

-18-

altered charged species of NGF. Incorrectly charged forms of NGF were purified away from the main NGF peak and concentrated.

Although the following examples describe each step of the process of the present invention for the production of biologically-active NGF, the present invention encompasses the production of biologically active neurotrophic factors from the NGF/BDNF family, including BDNF, NGF, NT3, and NT4, as well as neurotrophic factors having substantial homology and similar biological activity. The degree of homology existing between members of the NGF/BDNF family of neurotrophic factors, including amino acid sequence and location of disulfide bonds, suggests that these proteins have similar three-dimensional structures. Further, the problems associated with incorrect formation of disulfide bonds and the need for improved methods for refolding and renaturing the bacterially produced protein are similar for all members of the NGF/BDNF family of neurotrophic factors.

Example 1. Construction of a Synthetic NGF Gene and Expression in E. coli.

A synthetic NGF gene was designed to optimize the codons for expression in E. coli as well as create unique restriction sites to facilitate subsequent cloning steps.

The NGF gene was assembled in two pieces: 1) Section A - a 218 base pair (bp) BamHI-SalI piece of DNA consisting of 3 pairs of complementary oligonucleotides synthesized on an Applied Biosystems 380A DNA synthesizer; 2) Section B - a 168 bp SalI-KpnI piece of DNA consisting of 2 pairs of complementary synthetic oligonucleotides (Figure 3) (SEQ ID NO:5).

35

-19-

A. Assembly of the Sections. Each oligonucleotide was phosphorylated using T4 polynucleotide kinase. The phosphorylated oligonucleotides were then annealed to their complements by heating to 80°C and slow cooling to 35°C. The pairs of oligonucleotides (3 for Section A and 2 for Section B) were ligated and subsequently digested with BamHI-SalI (Section A) or SalI-KpnI (Section B) to minimize multiple insertions. The resulting fragments were isolated on 5% polyacrylamide gel and eluted. Each fragment was ligated into a pUC18 fragment digested with the appropriate enzymes (BamHI and SalI for Section A; SalI and KpnI for Section B). JM109 was transformed and isolates were grown in Luria broth with ampicillin added at a concentration of 100 µg/ml. Plasmid DNA was prepared and confirmed to have the appropriately sized fragment by restriction digest analysis.

B. Assembly of the Entire Synthetic NGF Gene. A BamHI-SalI 218 bp fragment was isolated from Section A pUC18 and a 168 bp SalI-KpnI fragment was isolated from Section B pUC18 as done above using a 5% polyacrylamide gel. These fragments were ligated into BamHI-KpnI cut pUC18 (IPTG and XGal were used to colorimetrically determine colonies with inserts with white colonies having inserts). A white colony was chosen and plasmid DNA prepared. When a BamHI-KpnI digest was done, a 387 bp fragment was released and isolated from a 1% agarose gel. The 387 bp fragment was ligated into a BamHI-KpnI, approximately 7 kilobase (Kb) vector fragment, REP pT3XI-2, obtained from a digest of plasmid TP NGF (2start-)REP pT2XI-2. REP is a repetitive extragenic palindrome sequence reported to stabilize messenger RNA by preventing 3'-5' exonucleolytic activity Merino et al. (1987) Gene 58:305). A transformant was grown in Luria broth and tetracycline at a concentration of 10 µg/ml. Plasmid DNA was prepared and digested with

-20-

BamHI-KpnI. A 387 bp fragment was observed but in order to ensure that the fragment was the synthetic gene, a second digest was done with BamHI-EcoRV (the EcoRV site is eliminated from the coding region of the synthetic gene). When the construct was sequenced, three areas appeared to have the wrong sequence and were corrected through in vitro mutagenesis using a Mutagene kit purchased from Biorad. The areas of concern were between nucleotides 83-91 (Mut1) (Figure 4, SEQ ID NO:6), nucleotide 191 (Mut2) (Figure 4, SEQ ID NO:7), and a deletion of 2 C's at nucleotides 258 and 259 (Mut3) (Figure 4, SEQ ID NO:8). The synthetic NGF gene was ligated into BamHI-KpnI cut mp18 as a BamHI-KpnI fragment to use as a template for the mutagenesis. The mutagenesis was done in a 2 step process, first using oligonucleotides Mut1 and Mut2 for a double mutagenesis. Two isolates with the correct sequence were chosen by hybridization to ³²P-labelled Mut1 and Mut2 oligonucleotides (called Mut1,2A and Mut1,2B). These were then mutagenized with Mut3 oligonucleotide in a second step and one isolate from each plate was chosen by hybridization to a ³²P-labelled Mut3 probe (Syn NGF MutA and Syn NGF MutB). Both isolates had the correct sequence. Replicative form (RF - double stranded DNA) was prepared and digested with BamHI-KpnI and a 387 bp fragment isolated from a 1% agarose gel. The Syn NGF MutA fragment was ligated into a 7 Kb BamHI-KpnI vector fragment, REP pT3XI-2, isolated from TP NGF REP pT3XI-2. MCB00005 was transformed, one isolate grown in Luria broth plus tetracycline at a concentration of 10 µg/ml, and plasmid DNA was prepared. A diagnostic BamHI-KpnI digest was done to confirm the presence of the insert.

C. Enhanced Synthetic NGF. As a means of boosting expression of the synthetic gene, the region between the initiating methionine and BamHI site was altered

-21-

through in vitro mutagenesis to resemble that of a highly expressed T7 bacteriophage protein, gene 10, using the oligonucleotide, Syn NGF 5P (Figure 5) (SEQ ID NO:9). Syn NGF MutA mp18 was used as the template.

5 Four plaques were chosen by hybridizing to a ³²P-labelled Syn NGF 5P oligonucleotide. RF DNA was made and digested with BamHI-KpnI. All had the appropriately sized fragment and the sequence of each was also correct. The BamHI-KpnI 387 bp fragment was
10 isolated from a 1% agarose gel and ligated into a BamHI-KpnI, approximately 7 Kb vector fragment, REP pT3XI-2 (isolated from BamHI-KpnI digested Syn NGF A REP pT3XI-2 described above). MCB00005 was transformed. Colonies were screened with ³²P-labelled
15 Syn NGF 5P oligonucleotide and 2 colonies that hybridized to the probe were grown in Luria broth plus tetracycline at a concentration of 10 µg/ml. Both isolates had the correct sequence.

20 D. Construction of TP NGF REP pT3XI-2 and TP NGF (2start-) REP pT3XI-2.

(1) TP NGF REP PT3XI-2. DH5α carrying a BamHI-HindIII fragment of the British Biotechnology NGF gene (British Biotechnology, Limited, Oxford, England) was digested
25 with BamHI-HindIII, a 380 base pair fragment was isolated, and ligated into BamHI-HindIII digested mp18. The DNA was in vitro mutagenized in a 2-step process. The first step involved insertion of a BamHI site immediately 3' to the HindIII site at the 5' end of the
30 gene and also insertion of a Shine-Delgarno (S/D) sequence with "optimal" spacing for efficient expression between the S/D and initiating Met codon (see TP NGF oligonucleotide, Figure 6) (SEQ ID NO:10). A plaque was chosen after hybridizing to the ³²P-
35 labelled TP NGF oligonucleotide (Figure 6) (SEQ ID NO:10). A second round of mutagenesis was done using the oligonucleotide REP NGF (Figure 6) (SEQ ID NO:11).

-22-

A plaque was chosen through hybridization with ³²P labelled REP NGF oligonucleotide. RF DNA was prepared, an approximately 470 Kb BamHI-HindIII fragment isolated, and ligated into an approximately 7 Kb BamHI-HindIII vector fragment isolated from Bam TP Δ53 pT3XI-2 (Figure 7) (SEQ ID NO:12). Strain MCB00005 was transformed and an isolate chosen, sequenced, and found to have the correct sequence.

(2) TP NGF (2start-) pT3XI-2. In order to eliminate a putative second initiation region within the NGF gene, in vitro mutagenesis was done using the oligonucleotide, 2start- (Figure 8) (SEQ ID NO:13), and TP NGF REP mp18 as the template. An isolate that hybridized to ³²P-labelled 2start- oligonucleotide was sequenced and found to have the correct sequence. RF DNA was prepared, a BamHI-HindIII approximately 470 bp fragment was isolated from a 1% agarose gel, and ligated into an approximately 7 Kb BamHI-HindIII fragment, pT3XI-2 (isolated from Bam TP Δ53 pT3XI-2, see Figure 7 (SEQ ID NO:12)). MCB00005 was transformed, and the correct isolate determined through restriction digest analysis.

Example 2. Isolation of Biologically Active NGF.

A. Cell Lysis. The human recombinant NGF gene construct of Example 1 was expressed in E. coli cells grown in chemically defined medium at 33°C. A fresh or frozen slurry of cells was diluted with 50 mM Tris-HCl, 10 mM EDTA, pH 8.5, to a final solids concentration of about 20% (weight/volume). Cells were lysed using 4 passes through a Gaulin or Rannie homogenizer at a pressure of >8000 PSI. The lysate was passed through a cooling coil to maintain the temperature at less than 15°C.

B. Harvest and wash of cell solids containing NGF.
Cell solids were captured using a Westphalia

-23-

centrifuge. After the capture the percent of solids was determined. Sufficient cold wash/dilution buffer (20mM Tris-HCl, pH 7.5) was added to lower the percent solids to 5 percent. After gentle mixing the lysate went for a second pass through the centrifuge. The final "NGF washed cell suspension" was assayed for percent solids. Approximately 80 g of washed solids were recovered per Kg of starting cells. The "NGF washed solids suspension" was either used immediately or was frozen at -20°C for subsequent use.

C. Solubilization and chemical modification of NGF.

The NGF present in the washed solids was solubilized by the use of 8 M urea and a sulfitolysis mixture. This resulted in solubilized, denatured, chemically-modified NGF in which the cysteine residues are present as a cys-SO₃⁻ mixed disulfide.

Sufficient solid urea and water were added to the "NGF washed solids suspension" to achieve a final concentration of 8 M urea in a final volume equal to twice the volume of washed solids suspension used. After dissolution of the urea, the following final concentrations of reagents were added for the step of sulfitolysis: 10 mM Tris buffer, 100 mM sodium sulfite, 10 mM sodium tetrathionate. The mixture was brought to a final pH of about 7.5 with HCl and stirred at room temperature for at least about 2 hours.

D. Capture and purification of sulfonylated NGF.

Sulfonylated NGF was captured and purified from the sulfitolysis mixture by anion exchange chromatography. Several loading and eluting schemes were utilized.

In one embodiment of the invention, the sulfitolysis mixture was diluted 10-fold in Buffer A (8 M urea, 20 mM Tris-HCl, pH 9.0), and adjusted to a final pH of 9.0 with NaOH. This solution was applied to a column of Pharmacia Q-Sepharose big bead resin

-24-

equilibrated with Buffer A. A volume representing 25 grams of NGF washed solids was loaded per liter of resin. The column was washed with 3 column volumes of Buffer A. Sulfonlated NGF was eluted by lowering the pH using Buffer B (8 M urea, 36 mM MES, pH 6.0). In another embodiment of the invention, sulfonlated NGF was eluted with a linear gradient from Buffer A to Buffer B in about 10 column volumes.

In the preferred embodiment of the invention, an alternate loading procedure utilized diafiltration of the sulfitolysis mixture using either an Amicon S1Y10 or S10Y10 spiral wrap ultrafiltration cartridge. The mixture was diafiltered with about 4 volumes of Buffer A. This diafiltered sulfitolysis mixture was adjusted to pH 9.0 and applied directly to a column of Q-Sepharose big bead resin. A volume representing 125 grams of NGF washed solids was loaded per liter of resin. The column was washed and the sulfonlated NGF was eluted as described above.

The protein eluted from the columns by any of the above described methods was primarily sulfonlated NGF. The peak of eluted protein was pooled and the protein concentration was determined by absorbance at 280 nm using an extinction coefficient of 1.44. Column yields of up to about 5 mg of protein per gram of NGF washed solids were typical.

E. Refolding NGF. Q-Sepharose-purified, sulfonlated NGF may be refolded by several methods. In the preferred embodiment, sulfonlated NGF was refolded at a final protein concentration of 0.1 mg/ml. The required final refold volume was calculated based on the amount of protein to be refolded. Filtered, deionized 8M urea, polyethylene glycol 300 (PEG 300), dibasic potassium phosphate and water were combined in a carboy such that final concentrations of 5M urea, 20% PEG 300 and 100 mM dibasic potassium phosphate were

-25-

attained in the final refold volume. The solution was cooled to about 10°C. Sulfonylated NGF was gently added to a final concentration of 0.1 mg/ml. The pH of this mixture was brought to about 8.7 with 5 M HCl.

5 Stirring was halted and L-cysteine was added to a final concentration of 3 mM. The gas phase was sparged for about 5 minutes with a vigorous stream of argon, and the carboy was sealed. The solution was stirred until the L-cysteine was dissolved, and the carboy was stored

10 at about 10°C for about 4 days.

Refolding efficiency was studied with NGF protein concentrations ranging between 0.02 and 0.2 mg/ml. Refolding efficiency improved with decreasing protein concentration, however the required volumes and the

15 cost of the refold reagents precluded optimization based solely on yield. Urea concentrations between 4.5 and 5.5 M proved optimal for refold. Yields fell off sharply below about 4 M urea, while concentrations above 5.5 M proved impractical.

20 Optimal refold was achieved using about 20% PEG 300. PEG 200 functioned nearly as well as PEG 300. Lower levels of PEG 300, or of PEG 200, or replacement of the PEG with ethylene glycol led to much lower refold efficiencies.

25 Refolding efficiencies were examined with phosphate concentrations between zero and 0.5 M. NGF refolding exhibited a broad optimum between 100 and 200 mM phosphate. A comparison of monobasic sodium phosphate, dibasic sodium phosphate, monobasic

30 potassium phosphate, and dibasic potassium phosphate showed only minor differences in NGF refold efficiencies. Dibasic potassium phosphate was preferred for its starting pH in solution and for its increased solubility over the comparable sodium salt.

35 NGF refolded optimally at about 10°C, although temperatures between 4°C and 15°C worked nearly as well. Refold fell sharply above 15°C, with negligible

-26-

refold occurring at room temperature. Increases in the pH of the refold solution led to large changes in the rate of refolding. For example, at pH 8.3 refolding took 8-9 days to near completion, while at pH 8.7
5 refolding was near complete after 4 days. Higher pH was avoided due to the urea present in the refold solution, the length of the refold, and the increased susceptibility of proteins to carbamylation with increasing pH.

10 L-cysteine or cysteamine were used with equal effectiveness to initiate NGF refolding. Cysteamine hydrochloride was slightly less effective. A final concentration of about 3 mM L-cysteine was optimal. L-cysteine concentrations below 2 mM or above 5 mM lead
15 to a substantial decrease in refolding.

F. Capture of refolded NGF. Properly refolded NGF present in the refold solution was captured using cation exchange chromatography. The column size was
20 chosen based upon the ability of the column to handle the required flow rate and backpressure encountered when loading a large volume of a viscous refold mixture, rather than on the protein loading capacity of the resin. A typical 70-liter refold was captured
25 using a 750 ml resin bed volume.

In the preferred embodiment for the capture of refolded NGF, the carboy was opened after storage at approximately 10°C for 4 days, and the refold solution was brought to pH 5.0 with either 5 M HCl or with
30 acetic acid. This solution was applied to a column of Pharmacia SP-Sepharose big bead resin which had been equilibrated in 20 mM sodium acetate, pH 5.0. After loading, the column was washed with 4 column volumes of 20 mM sodium acetate, pH 5.0. Soluble, refolded NGF
35 was eluted from the column using 20 mM sodium acetate, 750 mM NaCl, pH 5.0. The flow rate was 0.5 column volumes per minute (c.v./min) during equilibration,

-27-

loading and washing, and was lowered to 0.25 c.v./min during elution.

5 A single peak of protein was recovered which was comprised of properly refolded NGF. Although this material appeared near homogeneous by sizing, SDS-PAGE, and standard reverse phase HPLC, it was subsequently shown to contain several altered charged species of NGF by isoelectric focusing and cation exchange HPLC. The principal species separated by cation exchange HPLC
10 were identified as truncated or carbamylated variants of NGF using electrospray mass spectroscopy. It thus became necessary to include an additional column in the purification process to remove these NGF variants.

15 G. Removal of NGF variants by ion exchange chromatography. In the preferred embodiment of this invention, the pool of protein eluted from the S-Sepharose big bead column was diluted two-fold using 20 mM sodium acetate, pH 5.0, and loaded onto a column of
20 Pharmacia SP-Sepharose high performance resin equilibrated in 20 mM sodium acetate, pH 5.0. The column was loaded to about 5 mg NGF/ml resin. The column was first washed with about 2 column volumes of 20 mM sodium acetate, pH 5.0, then with about 2 column
25 volumes of 20 mM Tris-HCl, 75 mM NaCl, pH 7.5, and eluted with a 12 column volumes linear gradient from 125 to 300 mM NaCl in 20 mM Tris-HCl, pH 7.5. In an alternate embodiment of the invention, the column was washed with about 3 column volumes of 20 mM sodium
30 phosphate buffer, 150 mM NaCl, pH 7.0. Protein was eluted from the column using a 10 column volumes pH gradient from 20 mM sodium phosphate, 150 mM NaCl, pH 7.0, to 20 mM Tris-HCl, 150 mM NaCl, pH 8.0. Both of the above elution schemes led to resolution of
35 incorrect charge forms of NGF away from the main peak of NGF.

Additional embodiments of the invention include

-28-

the use of other cation exchange resins, such as Pharmacia Mono-S, or elution of the protein with different buffer systems, and/or at a different pH. These included 20 mM glycylglycine, pH 8.5, 50-200 mM NaCl gradient; 20 mM borate, pH 8.0-8.5, 50-300 mM NaCl gradient; 20 mM Tris-HCl, pH 7.0-8.5, 50-400 mM NaCl gradient; and 20 mM sodium phosphate, pH 7.0, 100-500 mM NaCl gradient. NGF was also eluted from the above column using 20 mM sodium acetate buffer, pH 5.0, 450-800 mM NaCl gradient, and with 20 mM sodium citrate, pH 5.0, 400-700 mM NaCl gradient.

H. Concentration/Diafiltration. Fractions containing purified NGF from the SP-Sepharose HP column were pooled, concentrated and exchanged into a final bulk formulation containing citrate and NaCl at pH about 5.2 using an Amicon YM10 membrane in a stirred cell. This step was done at room temperature, with the protein concentration maintained below 5 mg/ml.

I. Precipitation of NGF. Purified NGF exhibited a tendency to precipitate under certain conditions. Factors which led to increased precipitation included increased protein concentration, increased NaCl concentration, increased pH, and decreased temperature. Thus, in the preferred embodiment of this invention, the protein concentration of NGF solutions are kept below 5 mg/ml, and the solutions are not cooled below about 10°C except to freeze the purified, formulated bulk.

Example 3. Determination of Biological Activity.

Biological activity of purified NGF was determined by testing its ability to promote the survival of chick embryo sympathetic chain neurons in vitro.

Cultures of chick embryo sympathetic chain and dorsal root ganglia were prepared as previously

-29-

described (Collins and Lile (1989) Brain Research 502:99). Briefly, sympathetic or dorsal root ganglia were removed from fertile, pathogen-free chicken eggs that had been incubated 8-11 days at 38°C in a humidified atmosphere. The ganglia were chemically dissociated by exposure first to Hanks' Balanced Salt Solution without divalent cations, containing 10 mM HEPES buffer pH 7.2 for 10 min at 37°C, then by exposure to a solution of 0.125% bacto trypsin 1:250 (Difco, Detroit, Michigan) in Hanks' Balanced Salt Solution modified as above for 12 min at 37°C. Trypsinization was stopped by addition of fetal calf serum to a final concentration of 10%. After this treatment, ganglia were transferred to a solution consisting of Dulbecco's high glucose Modified Eagle Medium without bicarbonate containing 10% fetal calf serum and 10 mM HEPES, pH 7.2 and mechanically dissociated by trituration approximately 10 times through a glass Pasteur pipet whose opening had been fire polished and constricted to a diameter such that it took 2 seconds to fill the pipet. The dissociated ganglia were then plated in culture medium (Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum, 4 mM glutamine, 60 mg/L penicillin-G, 25 mM HEPES, pH 7.2) in 100 mm diameter tissue culture dishes (40 dissociated ganglia per dish) for three hours. This preplating was done in order to separate the nonneuronal cells, which adhere to the dish, from the nerve cells, which do not adhere. After three hours, the nonadherent nerve cells were collected by centrifugation, resuspended in culture medium, and plated in 50 μ l per well onto half area 96 well microtiter tissue culture plates at a density of 1500 nerve cells per well. The microtiter wells had been previously exposed to a 1 mg/ml solution of poly-L-ornithine in 10 mM sodium borate, pH 8.4 overnight at 4°C, washed in distilled water, and air dried.

-30-

10 μ l of a serial dilution of the sample to be assayed for neurotrophic activity was added to each well and the dishes were incubated for 20 hours at 37°C in a humidified atmosphere containing 7.5% CO₂. After
5 18 hours, 15 μ l per well of a 1.5 mg/ml solution of the tetrazolium dye MTT in Dulbecco's high glucose modified Eagle Medium without bicarbonate containing 10 mM HEPES, pH 7.2 was added and the cultures placed back in the 37°C incubator for 4 hours. Then 75 μ l of a
10 solution of 6.7 ml of 12 M HCl per liter of isopropanol was added and the contents of each well triturated 30 times to break open the cells and suspend the dye. The absorbance at 570 nm was determined relative to a 690 nm reference for each well using an automatic
15 microtiter plate reader (Dynatech, Chantilly, Virginia). The absorbance of wells which had not received any neurotrophic agent (negative controls) was subtracted from the absorbance of sample-containing wells. The resulting absorbance is proportional to the
20 number of living cells in each well, defined as those nerve cells capable of reducing the dye. The concentration of trophic activity in trophic units (TU) per ml was defined as the dilution that gave 50% of maximal survival of nerve cells. For example, if the
25 sample gave 50% maximal survival when diluted 1:100,000 the titer was defined as 100,000 TU/ml. Specific activity was determined by dividing the number of trophic units per ml by the concentration of protein per ml in the undiluted sample.

30 Figure 9 sets forth the process of this invention in a flow diagram.

-31-

SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- (i) APPLICANT: Jack Lile
Tadahiko Kohno
Duane Bonam
Mary S. Rosendahl
 - (ii) TITLE OF INVENTION: Production of Biologically Active
Recombinant Neurotrophic Protein
 - (iii) NUMBER OF SEQUENCES: 13
 - (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Swanson & Bratschun, L.L.C.
 - (B) STREET: 8400 E. Prentice Avenue, Suite 200
 - (C) CITY: Englewood
 - (D) STATE: Colorado
 - (E) COUNTRY: USA
 - (F) ZIP: 80111
 - (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Diskette, 3.50 inch, 1.44 MG storage
 - (B) COMPUTER: IBM compatible
 - (C) OPERATING SYSTEM: MS-DOS
 - (D) SOFTWARE: WordPerfect 5.1
 - (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/266,090
 - (B) FILING DATE: 27-JUNE-1994
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/240,122
 - (B) FILING DATE: 09-MAY-1994
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 08/087,912
 - (B) FILING DATE: 06-JULY-1993
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 07/680,681
 - (B) FILING DATE: 04-APRIL-1991
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 07/594,126
 - (B) FILING DATE: 09-OCT-1990
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 07/547,750
 - (B) FILING DATE: 02-JULY-1990
 - (vii) PRIOR APPLICATION DATA:
 - (A) APPLICATION NUMBER: 07/505,441
 - (B) FILING DATE: 06-APRIL-1990
 - (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Barry J. Swanson
 - (B) REGISTRATION NUMBER: 33,215
 - (C) REFERENCE/DOCKET NUMBER: SYNE200/PCT
 - (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (303) 793-3333
 - (B) TELEFAX: (303) 793-3433
- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 742 base pairs

-32-

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: nucleic acid sequence for human BDNF

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGCATCCTT	TTCCTTACTA	TGGTTATTTT	ATACTTTGGT	TGCATGAAGG	50
CTGCCCCCAT	GAAAGAAGCA	AACATCCGAG	GACAAGGTGG	CTTGGCCTAC	100
CCAGGTGTGC	GGACCCATGG	GACTCTGGAG	AGCGTGAATG	GGCCCAAGGC	150
AGGTTCAAGA	GGCTTGACAT	CATTGGCTGA	CACTTTCGAA	CACGTGATAG	200
AAGAGCTGTT	GGATGAGGAC	CAGAAAGTTC	GGCCCAATGA	AGAAAAACAAT	250
AAGGACGCAG	ACTTGTACAC	GTCCAGGGTG	ATGCTCAGTA	GTCAAGTGCC	300
TTTGGAGCCT	CCTCTTCTCT	TTCTGCTGGA	GGAATACAAA	AATTACCTAG	350
ATGCTGCAAA	CATGTCCATG	AGGGTCCGGC	GCCACTCTGA	CCCTGCCCGC	400
CGAGGGGAGC	TGAGCGTGTG	TGACAGTATT	AGTGAGTGGG	TAACGGCGGC	450
AGACAAAAAG	ACTGCAGTGG	ACATGTCGGG	CGGGACGGTC	ACAGTCCTTG	500
AAAAGGTCCC	TGTATCAAAA	GGCCAACTGA	AGCAATACTT	CTACGAGACC	550
AAGTGCAATC	CCATGGGTTA	CACAAAAGAA	GGCTGCAGGG	GCATAGACAA	600
AAGGCATTGG	AACTCCCAGT	GCCGAAC TAC	CCAGTCGTAC	GTGCGGGCCC	650
TTACCATGGA	TAGCAGAAAG	AGAATTGGCT	GGCGATTCAT	AAGGATAGAC	700
ACTTCTTG TG	TATGTACATT	GACCATTAAA	AGGGGAAGAT	AG	742

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 725 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: nucleic acid sequence for human NGF

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGTCCATGT	TGTTCTACAC	TCTGATCACA	GCTTTTCTGA	TCGGCATACA	50
GGCGGAACCA	CACTCAGAGA	GCAATGTCCC	TGCAGGACAC	ACCATCCCCC	100
AAGTCCACTG	GAATAAACTT	CAGCATTTCC	TTGACACTGC	CCTTCGCAGA	150
GCCCGCAGCG	CCCCGGCAGC	GGCGATAGCT	GCACGCGTGG	CGGGGCAGAC	200
CCGCAACATT	ACTGTGGACC	CCAGGCTGTT	TAAAAAGCGG	CGACTCCGTT	250
CACCCCGTGT	GCTGTTTAGC	ACCCAGCCTC	CCCGTGAAGC	TGCAGACACT	300
CAGGATCTGG	ACTTCGAGGT	CGGTGGTGCT	GCCCCCTTCA	ACAGACTCAC	350
AGGAGCAAGC	GGTCATCATC	CCATCCCATC	TTCCACAGGG	GCGAATTCTC	400
GGTGTGTGAC	AGTGTGAGCG	TGTGGGTTGG	GGATAAGACC	ACCGCCACAG	450
ACATCAAGGG	CAAGGAGGTG	ATGGTGTGGG	GAGAGGTGAA	CATTAACAAC	500
AGTGTATTCA	AACAGTACTT	TTTTGAGACC	AAGTGCCGGG	ACCCAAATCC	550
CGTTGACAGC	GGGTGCCGGG	GCATTGACTC	AAAGCACTGG	AACTCATATT	600
GTACCACGAC	TCACACCTTT	GTCAAGGCGC	TGACCATGGA	TGGCAAGCAG	650
GCTGCCTGGC	GGTTTATCCG	GATAGATACG	GCCTGTGTGT	GTGTGCTCAG	700
CAGGAAGGCT	GTGAGAAGAG	CCTGA			725

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 247 amino acids
- (B) TYPE: amino acid
- (C) TOPOLOGY: linear

(ix) FEATURE:

- (A) NAME/KEY: inferred amino acid sequence of human BDNF

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Thr Ile Leu Phe Leu Thr Met Val Ile Ser Tyr Phe

-33-

	-125		-120
Gly Cys Met Lys Ala Ala Pro Met Lys Glu Ala Asn Ile			
-115	-110	-105	
Arg Gly Gln Gly Gly Leu Ala Tyr Pro Gly Val Arg Thr			
-100	-95	-90	
His Gly Thr Leu Glu Ser Val Asn Gly Pro Lys Ala Gly			
	-85	-80	
Ser Arg Gly Leu Thr Ser Leu Ala Asp Thr Phe Glu His			
-75	-70	-65	
Val Ile Glu Glu Leu Leu Asp Glu Asp Gln Lys Val Arg			
	-60	-55	
Pro Asn Glu Glu Asn Asn Lys Asp Ala Asp Leu Tyr Thr			
-50	-45	-40	
Ser Arg Val Met Leu Ser Ser Gln Val Pro Leu Glu Pro			
-35	-30	-25	
Pro Leu Leu Phe Leu Leu Glu Glu Tyr Lys Asn Tyr Leu			
	-20	-15	
Asp Ala Ala Asn Met Ser Met Arg Val Arg Arg His Ser			
-10	-5	1	
Asp Pro Ala Arg Arg Gly Glu Leu Ser Val Cys Asp Ser			
5	10	15	
Ile Ser Glu Trp Val Thr Ala Ala Asp Lys Lys Thr Ala			
	20	25	
Val Asp Met Ser Gly Gly Thr Val Thr Val Leu Glu Lys			
30	35	40	
Val Pro Val Ser Lys Gly Gln Leu Lys Gln Tyr Phe Tyr			
	45	50	
Glu Thr Lys Cys Asn Pro Met Gly Tyr Thr Lys Glu Gly			
55	60	65	
Cys Arg Gly Ile Asp Lys Arg His Trp Asn Ser Gln Cys			
70	75	80	
Arg Thr Thr Gln Ser Tyr Val Arg Ala Leu Thr Met Asp			
	85	90	
Ser Arg Lys Arg Ile Gly Trp Arg Phe Ile Arg Ile Asp			
95	100	105	
Thr Ser Cys Val Cys Thr Leu Thr Ile Lys Arg Gly Arg			
	110	115	

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 241 amino acids

(B) TYPE: amino acid

(C) TOPOLOGY: linear

(ix) FEATURE:

(A) NAME/KEY: inferred amino acid sequence of human NGF

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ser Met Leu Phe Tyr Thr Leu Ile Thr Ala Phe Leu	
-120	-115
Ile Gly Ile Gln Ala Glu Pro His Ser Glu Ser Asn Val	
-105	-100
Pro Ala Gly His Thr Ile Pro Gln Val His Trp Thr Lys	
-95	-90
Leu Gln His Ser Leu Asp Thr Ala Leu Arg Arg Ala Arg	
-80	-75
Ser Ala Pro Ala Ala Ala Ile Ala Ala Arg Val Ala Gly	
-65	-60

Gln	Thr	Arg	Asn	Ile	Thr	Val	Asp	Pro	Arg.	Leu	Phe	Lys
-55						-50					-45	
Lys	Arg	Arg	Leu	Arg	Ser	Pro	Arg	Val	Leu	Phe	Ser	Thr
			-40					-35				
Gln	Pro	Pro	Arg	Glu	Ala	Ala	Asp	Thr	Gln	Asp	Leu	Asp
-30					-25					-20		
Phe	Glu	Val	Gly	Gly	Ala	Ala	Pro	Phe	Asn	Arg	Thr	His
	-15						-10					-5
Arg	Ser	Lys	Arg	Ser	Ser	Ser	His	Pro	Ile	Phe	His	Arg
				1				5				
Gly	Glu	Phe	Ser	Val	Cys	Asp	Ser	Val	Ser	Val	Trp	Val
10					15					20		
Gly	Asp	Lys	Thr	Thr	Ala	Thr	Asp	Ile	Lys	Gly	Lys	Glu
		25					30					35
Val	Met	Val	Leu	Gly	Glu	Val	Asn	Ile	Asn	Asn	Ser	Val
				40					45			
Phe	Lys	Gln	Tyr	Phe	Phe	Glu	Thr	Lys	Cys	Arg	Asp	Pro
	50					55					60	
Asn	Pro	Val	Asp	Ser	Gly	Cys	Arg	Gly	Ile	Asp	Ser	Lys
			65					70				
His	Trp	Asn	Ser	Tyr	Cys	Thr	Thr	Thr	His	Thr	Phe	Val
75					80					85		
Lys	Ala	Leu	Thr	Met	Asp	Gly	Lys	Gln	Ala	Ala	Trp	Arg
		90					95					100
Phe	Ile	Arg	Ile	Asp	Thr	Ala	Cys	Val	Cys	Val	Leu	Ser
				105					110			
Arg	Lys	Ala	Val	Arg	Arg	Ala						
	115					120						

80 TCT AAA CAC TGG AAC TCC TAC TGC ACC ACT ACT CAC 278
GAP Ser Lys His Trp Asn Ser Tyr Cys Thr Thr Thr His

- 35 -

[illegible]

(2) INFORMATION FOR SEQ ID NO:6:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 32 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
 CGATTCGGTT AGCGTTTGGG TTGGCGACAA AA

(2) INFORMATION FOR SEQ ID NO:7:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 21 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:
 CTTCTTGGA ACCAAATCCC G

(2) INFORMATION FOR SEQ ID NO:8:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 22 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
 CACTGGAACCT CCACTGCAC CA 22

(2) INFORMATION FOR SEQ ID NO:9:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 35 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:
 GGATCCAAGA AGGAGATATA CATATGTCTA GCAGC

(2) INFORMATION FOR SEQ ID NO:10:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 43 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:
 TGCCAAGCTT GGATCCAAGA AGGAGATATA CATATGTCAT CAT

-36-

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 119 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGCTGTGAGA TAAGGTACCG ACATTGCCTG ATGGCGCTGT GCGTGTCAGG	50
CCTACGGGGA GCATCGTAGG CCGGGCAAGG CACAGCCGCC ATCCGGCGTG	100
CCCAAGCTTG GATCCCCGG	119

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 691 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCTGTTGACA ATTAATCATC GGCTCGTATA ATGTGTGGAA TTGTGAGCGG	50
ATAACAATTT CACACAGGAA ACAGAATTCC ACAACGGTTT CCCTCTAGAA	100
ATAATTTTGT TTGGATCCAA GAAGGAGATA TACAT ATG TTG CCC GCC	147
Met Leu Pro Ala	
1	
CAG GTG GCA TTT ACA CCC TAC GCT CCG GAA CCG GGT TCT ACC	189
Gln Val Ala Phe Thr Pro Tyr Ala Pro Glu Pro Gly Ser Thr	
5 10 15	
TGC CGG CTC AGA GAA TAC TAT GAC CAG ACA GCT CAG ATG TGC	231
Cys Arg Leu Arg Glu Tyr Tyr Asp Gln Thr Ala Gln Met Cys	
20 25 30	
TGC AGC AAG TGC TCG CCG GGC CAA CAT GCA AAA GTC TTC TGT	273
Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys Val Phe Cys	
35 40 45	
ACC AAG ACC TCG GAC ACC GTG TGT GAC TCC TGT GAG GAC AGC	315
Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp Ser	
50 55 60	
ACA TAC ACC CAG CTC TGG AAC TGG GTT CCC GAG TGC TTG AGC	357
Thr Tyr Thr Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser	
65 70 75	
TGT GGC TCC CGC TGT AGC TCT GAC CAG GTG GAA ACT CAA GCC	399
Cys Gly Ser Arg Cys Ser Ser Asp Gln Val Glu Thr Gln Ala	
80 85	
TGC ACT CGG GAA CAG AAC CGC ATC TGC ACC TGC AGG CCC GGC	441
Cys Thr Arg Glu Gln Asn Arg Ile Cys Thr Cys Arg Pro Gly	
90 95 100	
TGG TAC TGC GCG CTG AGC AAG CAG GAG GGG TGC CGG CTG TGC	483
Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys Arg Leu Cys	
105 110 115	
GCG CCG CTG CGC AAG TGC CGC CCG GGC TTC GGC GTG GCC AGA	525
Ala Pro Leu Arg Lys Cys Arg Pro Gly Phe Gly Val Ala Arg	
120 125 130	
CCA GGA ACT GAA ACA TCA GAC GTG GTG TGC AAG CCC TGT GCC	567
Pro Gly Thr Glu Thr Ser Asp Val Val Cys Lys Pro Cys Ala	
135 140 145	
CCG GGG ACG TTC TCC AAC ACG ACT TCA TCC ACG GAT ATT TGC	609
Pro Gly Thr Phe Ser Asn Thr Thr Ser Ser Thr Asp Ile Cys	
150 155	

-37-

AGG	CCC	CAC	CAG	ATC	TGT	AAC	GTG	GTG	GCC	ATC	CCT	GGG	AAT	651
Arg	Pro	His	Gln	Ile	Cys	Asn	Val	Val	Ala	Ile	Pro	Gly	Asn	
160					165					170				
GCA	AGC	AGG	GAT	GCA	GTC	TGC	ACG	TCC	ACG	TCC	TAA	GCTT	691	
Ala	Ser	Arg	Asp	Ala	Val	Cys	Thr	Ser	Thr	Ser				
175					180									

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 33 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TCAAGGGCAA AGAAGTGATG GTATTGGGAG AGG 33

-38-

CLAIMS

1. A process for the production of biologically active recombinant neurotrophic factor wherein said neurotrophic factor is selected from the group
5 consisting of NGF, BDNF, NT3, or NT4, comprising:
 - a) expressing a gene coding for the neurotrophic factor in a bacterial expression system wherein said neurotrophic factor protein is produced;
 - b) solubilizing said neurotrophic factor in
10 urea;
 - c) sulfonylating said neurotrophic factor;
 - d) isolating and purifying the sulfonylated neurotrophic factor;
 - e) allowing the sulfonylated neurotrophic factor
15 to refold to give the biologically active neurotrophic factor; and
 - f) purifying the biologically active neurotrophic factor.
- 20 2. The process of claim 1 wherein said neurotrophic factor gene is comprised of DNA coding for human NGF.
- 25 3. The process of claim 1 wherein said neurotrophic factor gene is comprised of DNA coding for animal NGF.
- 30 4. The process of claim 1 wherein the neurotrophic factor gene is comprised of the sequence of Figure 1 (SEQ ID NO:1).
- 35 5. The process of claim 1 wherein the neurotrophic gene is comprised of DNA coding for human BDNF.
6. The process of claim 1 wherein the neurotrophic gene is comprised of the sequence of

-39-

Figure 1 (SEQ ID NO:2).

7. The process of claim 1 wherein said neurotrophic factor is solubilized with 8M urea.

5

8. The process of claim 1 wherein said neurotrophic factor refolds in the presence of polyethylene glycol (PEG) with a molecular weight of between 200-300.

10

9. The refolding step of claim 8 wherein PEG is present in the concentration of between 15-20% (weight/volume), urea is present in the concentration range of 4.5 - 5.5 M, the final protein concentration of neurotrophic factor is about 0.1 mg/ml, and the refolding step takes place at a temperature of about 10°C.

15

10. The refolding step of claim 1, 7, or 8 wherein refolding is initiated with the addition of one of L-cysteine or cysteamine.

20

11. The process of claim 1 wherein the sulfonylated neurotrophic factor is isolated and purified by anion exchange chromatography.

25

12. The process of claim 1 wherein sulfonylated neurotrophic factor is isolated and purified utilizing concentration and diafiltration.

30

13. The process of claim 1 wherein biologically active neurotrophic factor is purified by ion exchange chromatography.

35

14. A process for the production of biologically active recombinant neurotrophic factor, wherein said neurotrophic factor is selected from the group

-40-

consisting of NGF, BDNF, NT3, or NT4, comprising:

- a) constructing a synthetic neurotrophic factor DNA gene for directing an E. coli expression system to produce a neurotrophic factor;
- 5 b) expressing said neurotrophic factor in the E. coli expression system;
- c) solubilizing and sulfonylating said neurotrophic factor;
- d) refolding sulfonylated neurotrophic factor
10 such that the correct tertiary structure necessary for full biological activity is obtained; and
- e) purifying the fully biologically active neurotrophic factor.

15 15. The process of claim 14 wherein said neurotrophic factor gene is comprised of DNA coding for human NGF.

20 16. The process of claim 14 wherein said neurotrophic factor gene is comprised of DNA coding for animal NGF.

25 17. The process of claim 14 wherein the neurotrophic factor gene is comprised of the sequence of Figure 1 (SEQ ID NO:1).

30 18. The process of claim 14 wherein the neurotrophic gene is comprised of DNA coding for human BDNF.

 19. The process of claim 14 wherein the neurotrophic gene is comprised of the sequence of Figure 1 (SEQ ID NO:2).

35 20. The process of claim 14 wherein said neurotrophic factor is solubilized with 8M urea.

-41-

21. The process of claim 14 wherein said neurotrophic factor refolds in the presence of polyethylene glycol (PEG) with a molecular weight of between 200-300.

5

22. The refolding step of claim 21 wherein PEG is present in the concentration of about 20% (weight/volume), urea is present in the concentration range of 4.5 - 5.5 M, the final protein concentration of neurotrophic factor is about 0.1 mg/ml, and the refolding step takes place at a temperature of about 10°C.

10

23. The refolding step of claim 14, 20, or 21 wherein refolding is initiated with the addition of one of L-cysteine or cysteamine.

15

24. The process of claim 14 wherein the sulfonylated neurotrophic factor is isolated and purified by anion exchange chromatography.

20

25. The process of claim 14 wherein sulfonylated neurotrophic factor is isolated and purified utilizing concentration and diafiltration.

25

26. The process of claim 14 wherein biologically active neurotrophic factor is purified by ion exchange chromatography.

FIGURE 1

bdnf ATG CATCCTTTTCCCTTACTATGGTTATTTTCATACTTTTGGTTGCATGAAGGCTGCCCCCATGAAAGA
ngf ATGTCCATGTTGTTCTACACTCTGATCACAGCTTTTCTGATCGGCATACAGGCGGAACCACTCAGA
AGCAAAACATCCGAGGACAAAGGT-----GGCTTGGCCCTACCCAGGCTGTGCGG---ACC-----CATGGGACTCT
GAGCAATGTCCCTGCA---GGA---CAC-----ACCATCCCCCAAGTCCACTGGACTAAACTTTCAGCAATTCCCT
GGAGAGC-----GTGAATGGGCCCCAAGCAGGTTCAAGAGGCTTGACATCATTTGGCTGACACTTTCGAACACAGT
TGAC-----ACTGCC---CTTCGAGAGCC---CGCAGCGCC---CCG---GCAGCGCGATAGCTGCACGCGT
GATAGAAGAGCTGTTGGATGAGGAC---CAGAAAGTTGGGCCCAATGAAGAAAAC-----AATAAGGACGCAGA
GGCGGG---CAGACCCGC---AACATT---ACTGTG-----GACCCAGGCTGTTT---AAAAAGCGGCG
CTTGTAACAGTCCAGGGTGATGCTCAGTAGTCAAGTGCCTTTTGGAGCCTCCTCTCTCTTTCTGCTGGAGGAATA
ACTCCGTTCAACCCCGTGCTGTTTAGCACCCAGCCTCCCGTGAAGCTGCAGACACTCAGGATCTGGACTTCGA
CAAAAATTACCTAGATGCTGCAAAACATGTCCATGAGGTCGGGCGC-----CACTCTGACCCCTGCCCGCGAGG
GGTCGGTGGTGCTGCCCCCTTCAACAGGACTCAAGAGCAAGCGGTCACTATCCCATCCCCTCTTCCACAGGGG
GGAGCTGAGCGGTGTGACAGTATTAGTGAGTGGTTAACGGCGGCAGACAAAAGACTGCAGTGGACATGTGCGG
CGAATTCTCGGTGTGTGACAGTGTACGCGTGTGGTTGGG-----GATAAGACCAACCGCCACAGACATCAAGGG
CGGACCGGTACAGTCCCTTGAAGAGTCCCTGTATCAAAAGGCCAACTGAAGCAATACCTTCTACGAGACCAAGTG
CAAGGAGGTGATGGTGTGGGAGAGGTGAACATTAAACAGTGTATTCAACAGTACTTTTGTGAGACCAAGTG
CAATCCCCATGGGTTACACAAAAGAAGGCTGCAGGGGCATAGACAAAAGGCATTGGAACTCCAGTGCAGGAACTAC
CCGGGACCCAAATCCCGTTGACAGCGGGTGCCGGGCATTGACTCAAAGCACTGGAACTCATATTGTATCCACGAC
CCAGTCGTACGTGCGGGCCCTTACCATGGATAGCAGAAAGAAATTTGGCTGGCGATTTCATAAGGATAGACACTTC
TCACACCTTTGTCAAGGCGCTGACCATGGATGCG---AAGCAGGCTGCCTGGCGGTTTATCCGGATAGATACGGC
TTGTGTATGTACATTGACCATTAAGGGGAAGATAG bdnf (SEQ ID NO:1)
CTGTGTGTGTGCTCAGCAGGAAGGCTGTGAGAAGAGCCTGA ngf (SEQ ID NO:2)

2/13

FIGURE 2

bdnf	Met	Thr	Ile	Leu	Phe	Leu	Thr	Met	Val	Ile	Ser	Tyr	Phe	Gly	Cys	Met	Lys
ngf	Met	Ser	Met	Leu	Phe	Tyr	Thr	Leu	Ile	Thr	Ala	Phe	Leu	Ile	Gly	Ile	Gln
Ala	Ala	Pro	Met	Lys	Glu	Ala	Asn	Ile	Arg	Gly	Gln	Gly	---	---	Gly	Leu	Ala
Ala	Glu	Pro	His	Ser	Glu	Ser	Asn	Val	Pro	Ala	---	Gly	---	His	---	---	Thr
Pro	Gly	Val	Arg	---	Thr	---	---	His	Gly	Thr	Leu	Glu	Ser	---	---	Val	Asn
Pro	Gln	Val	His	Trp	Thr	Lys	Leu	Gln	His	Ser	Leu	Asp	---	---	Thr	Ala	---
Pro	Lys	Ala	Gly	Ser	Arg	Gly	Leu	Thr	Ser	Leu	Ala	Asp	Thr	Phe	Glu	His	Val
Arg	Arg	Ala	---	Arg	Ser	Ala	---	Pro	---	Ala	Ala	Ile	Ala	Ala	Arg	Val	Ala
Glu	Glu	Leu	Leu	Asp	Glu	Asp	---	Gln	Lys	Val	Arg	Pro	Asn	Glu	Glu	Asn	---
Gly	---	Gln	Thr	Arg	---	Asn	Ile	---	Thr	Val	---	---	---	Asp	Pro	Arg	Leu
Asn	Lys	Asp	Ala	Asp	Leu	Tyr	Thr	Ser	Arg	Val	Met	Leu	Ser	Ser	Gln	Val	Pro
---	Lys	Lys	Arg	Arg	Leu	Arg	Ser	Pro	Arg	Val	Val	Phe	Ser	Thr	Gln	Pro	Pro
Glu	Pro	Pro	Leu	Leu	Phe	Leu	Leu	Glu	Glu	Tyr	Lys	Asn	Tyr	Leu	Asp	Ala	Ala
Glu	Ala	Ala	Asp	Thr	Gln	Asp	Leu	Asp	Phe	Glu	Val	Gly	Gly	Ala	Ala	Pro	Phe
Met	Ser	Met	Arg	Val	Arg	Arg	---	---	His	Ser	Asp	Pro	Ala	Arg	Arg	Gly	Leu
Arg	Thr	His	Arg	Ser	Lys	Arg	Ser	Ser	Ser	His	Pro	Ile	Phe	His	Arg	Gly	Phe
Ser	Val	Cys	Asp	Ser	Ile	Ser	Glu	Trp	Val	Thr	Ala	Ala	Asp	Lys	Lys	Thr	Ala
Ser	Val	Cys	Asp	Ser	Val	Ser	Val	Trp	Val	Gly	---	---	Asp	Lys	Thr	Thr	Ala
Asp	Met	Ser	Gly	Gly	Thr	Val	Thr	Val	Leu	Glu	Lys	Val	Pro	Val	Ser	Lys	Gln
Asp	Ile	Lys	Gly	Lys	Glu	Val	Met	Val	Leu	Gly	Glu	Val	Asn	Ile	Asn	Asn	Ser
Leu	Lys	Gln	Tyr	Phe	Tyr	Glu	Thr	Lys	Cys	Asn	Pro	Met	Gly	Tyr	Thr	Lys	Glu
Phe	Lys	Gln	Tyr	Phe	Phe	Glu	Thr	Lys	Cys	Arg	Asp	Pro	Asn	Pro	Val	Asp	Ser

3/13

FIGURE 2 (CONT'D)

Cys Arg Gly Ile Asp Lys Arg His Trp Asn Ser Gln Cys Arg Thr Thr Gln Ser Tyr	
Cys Arg Gly Ile Asp Ser Lys His Trp Asn Ser Tyr Cys Thr Thr Thr His Thr Phe	
Val Arg Ala Leu Thr Met Asp Ser Arg Lys Arg Ile Gly Trp Arg Phe Ile Arg Ile	
Val Lys Ala Leu Thr Met Asp Gly --- Lys Gln Ala Ala Trp Arg Phe Ile Arg Ile	
Asp Thr Ser Cys Val Cys Thr Leu Thr Ile Lys Arg Gly Arg (SEQ ID NO:3)	
Asp Thr Ala Cys Val Cys Val Cys Thr Arg Lys Ala Val Arg Arg Ala (SEQ ID NO:4)	

5/13

FIGURE 3 (CONT'D)

Sal I
 ACCCGGTCGACTCGGCTGCCGGTATCGACTCTAAACACTGGAACCTCTACTGCACCCTACTACACAC 280
 TGGGCCAGCTGAGGCCGACGGCACCATAGCTGAGATTGTGACCTTGAGGATGACGTTGGTGAATGAGTGTG
 Asn Pro Val Asp Ser Gly Cys Arg Gly Ile Asp Ser Lys His Trp Asn Ser Tyr Cys Thr Thr His Thr
 CTTCGTTAAAGCTCTGACCATGGACGGCAACAGGCTGCATGGCGTTTATTTCGTATTGACACCGCATGT 350
 GAAGCAATTTTCGAGACTGGTACCTGCCGTTTGTCCGACGTACCGCAAAATAAGCATAACTGTGGCGGTACA
 Phe Val Lys Ala Leu Thr Met Asp Gly Lys Gln Ala Ala Trp Arg Phe Ile Arg Ile Asp Thr Ala Cys
 Kpn I
 GTTTGGGTACTGAGCCGCAAGCTGTTCGTTAAGGTACC 389
 CAAACGCATGACTCGGCGTTTCGACAAGCAATTCATGG
 Val Cys Val Leu Ser Arg Lys Ala Val Arg • (SEQ ID NO:5)

6/13

FIGURE 4

NGF Mut 1
5'-CGA TTC CGT TAG C[GT TTG GGT T]GG CGA CAA AA-3' 32
(SEQ ID NO:6)
Tm=96°C

NGF Mut 2
5'-CTT CTT CGA A[A]C CAA ATG CCG-3' 21
(SEQ ID NO:7)
Tm=62°C

NGF Mut 3
5'-CAC TGG AAC T[CC] TAC TGC ACC A-3' 22
(SEQ ID NO:8)
Tm 48+20=68°C

7/13

FIGURE 5

Syn NGF 5P

5'-GGA TCC AAG AAG GAG ATA TAC ATA TG TCT AGC AGC -3' 35
(SEQ ID NO:9)

FIGURE 6

1. TP NGF

5'-TGC CAA GCT TGG ATC CAA GAA GGA GAT ATA CAT ATG TCA TCA T-
3' 43
(SEQ ID NO:10)

2. REP NGF

5'-GGC TGT GAG ATA AGG TAC CGA CAT TGC CTG ATG GCG CTG TGC GTG
TCA GGC CTA CGG GGA GCA TCG TAG GCC GGG CAA GGC ACA GCC GCC
ATC CGG CGT GCC CAA GCT TGG ATC CCC GG-3' 119
(SEQ ID NO:11)

8/13

FIGURE 7

BamTP Δ 53 Sequence (EcoRI, BamHI, and HindIII sites highlighted)
(SEQ ID NO:12)

```

GCT GTT GAC AAT TAA TCA TCG GCT CGT ATA ATG TGT GGA ATT GTG AGC GGA TAA
                                     EcoRI
CAA TTT CAC ACA GGA AAC AGA ATT CCA CAA CGG TTT CCC TCT AGA AAT AAT TTT GTT TGG
                                     BamHI
-----
ATC CAA GAA GGA GAT ATA CAT ATG TTG CCC GCC CAG GTG GCA TTT ACA
      Met Leu Pro Ala Gln Val Ala Phe Thr

CCC TAC GCT CCG GAA CCG GGT TCT ACC TGC CGG CTC AGA GAA TAC TAT GAC CAG
Pro Tyr Ala Pro Glu Pro Gly Ser Thr Cys Arg Leu Arg Glu Tyr Tyr Asp Gln

ACA GCT CAG ATG TGC TGC AGC AAG TGC TCG CCG GGC CAA CAT GCA AAA GTC TTC
Thr Ala Gln Met Cys Cys Ser Lys Cys Ser Pro Gly Gln His Ala Lys Val Phe

TGT ACC AAG ACC TCG GAC ACC GTG TGT GAC TCC TGT GAG GAC AGC ACA TAC ACC
Cys Thr Lys Thr Ser Asp Thr Val Cys Asp Ser Cys Glu Asp Ser Thr Tyr Thr

CAG CTC TGG AAC TGG GTT CCC GAG TGC TTG AGC TGT GGC TCC CGC TGT AGC TCT
Gln Leu Trp Asn Trp Val Pro Glu Cys Leu Ser Cys Gly Ser Arg Cys Ser Ser

GAC CAG GTG GAA ACT CAA GCC TGC ACT CGG GAA CAG AAC CGC ATC TGC ACC TGC
Asp Gln Val Glu Thr Gln Ala Cys Thr Arg Glu Gln Asn Arg Ile Cys Thr Cys

AGG CCC GGC TGG TAC TGC GCG CTG AGC AAG CAG GAG GGG TGC CGG CTG TGC GCG
Arg Pro Gly Trp Tyr Cys Ala Leu Ser Lys Gln Glu Gly Cys Arg Leu Cys Ala

CCG CTG CGC AAG TGC CGC CCG GGC TTC GGC GTG GCC AGA CCA GGA ACT GAA ACA
Pro Leu Arg Lys Cys Arg Pro Gly Phe Val Ala Arg Pro Gly Thr Glu Thr

```

9/13

FIGURE 7 (CONT'D)

TCA GAC GTG GTG TGC TGC AAG CCC TGT GCC CCG GGG ACG TTC TCC AAC ACG ACT TCA	
Ser Asp Val Val Cys Lys Pro Cys Ala Pro Gly Thr Phe Ser Asn Thr Thr Ser	
TCC ACG GAT ATT TGC AGG CCC CAC CAG ATC TGT AAC GTG GTG GCC ATC CCT GGG	
Ser Thr Asp Ile Cys Arg Pro His Gln Ile Cys Asn Val Val Ala Ile Pro Gly	
	HindIII

AAT GCA AGC AGG GAT GCA GTC TGC ACG TCC ACG TCC TAA GCTT	
Asn Ala Ser Arg Asp Ala Val Cys Thr Ser Thr Ser	

10 / 13

FIGURE 8

2 Start (-)

(SEQ ID NO:13)

5' -TCA AGG GCA AAG AAG TGA TGG TAT TGG GAG AGG-3'

30

11 / 13

FIGURE 9

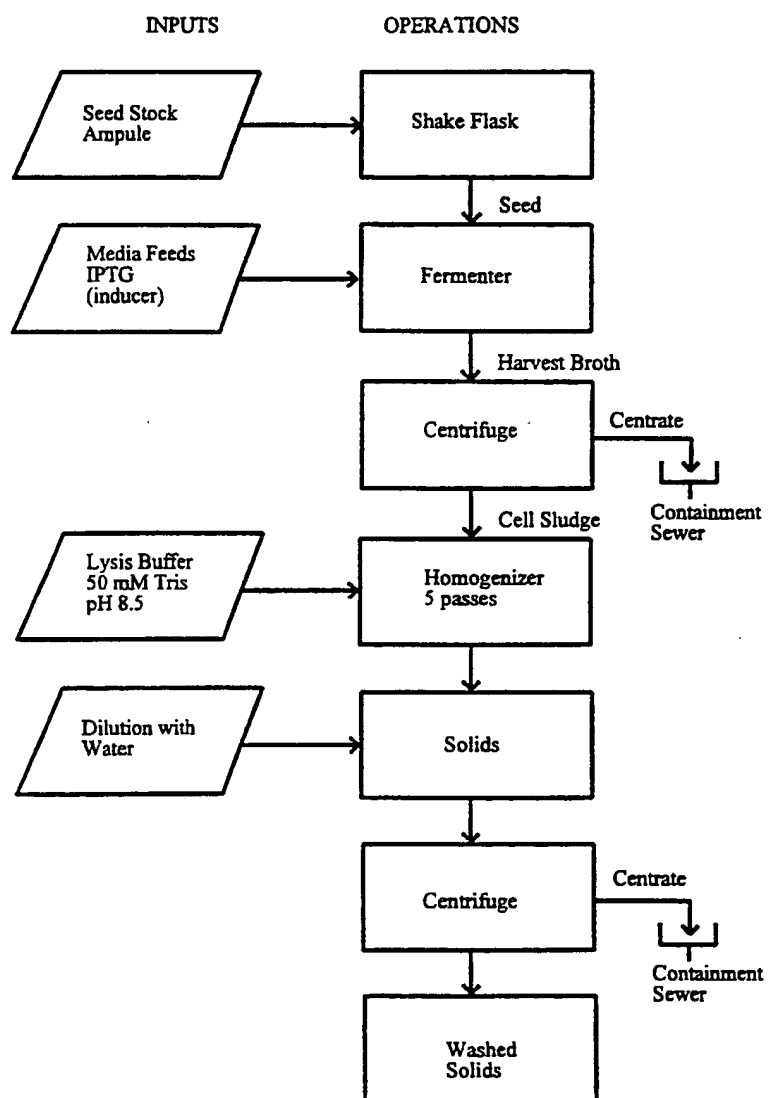
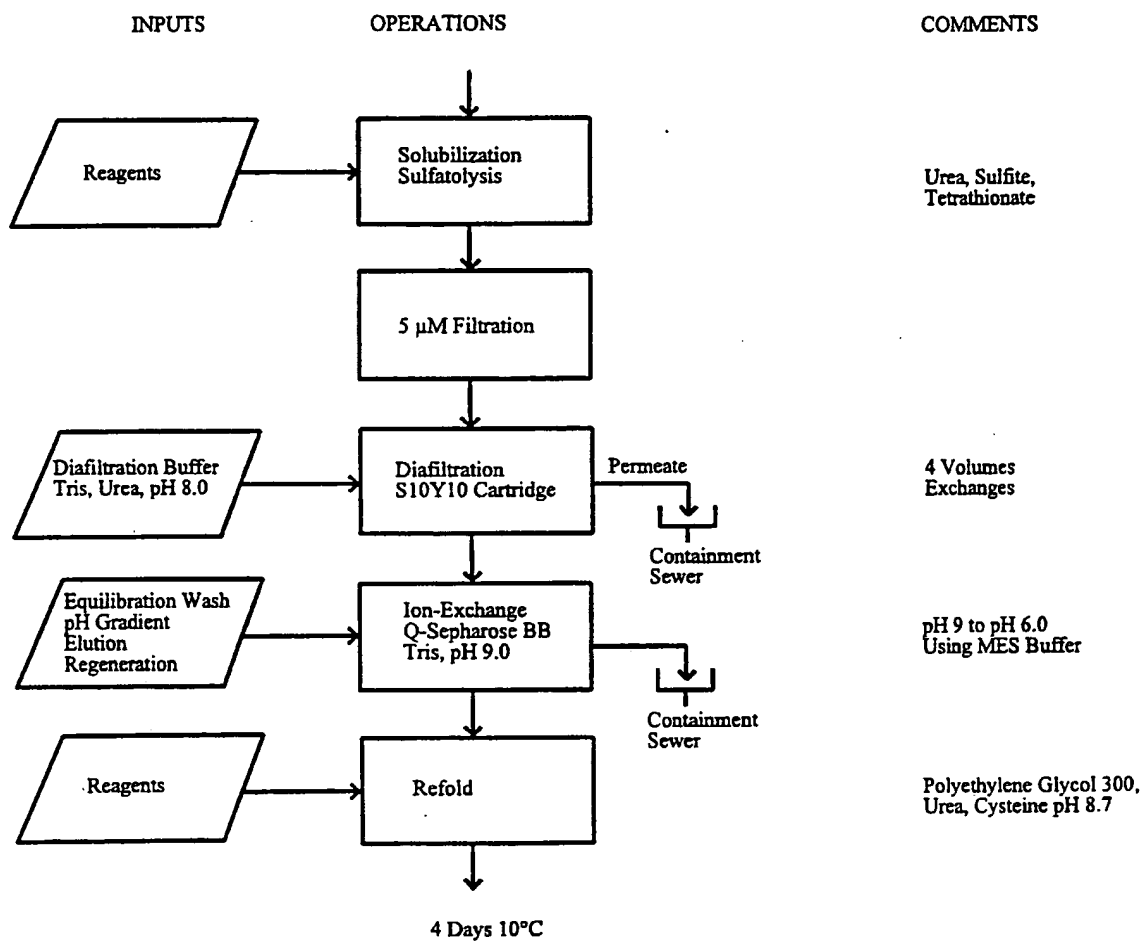
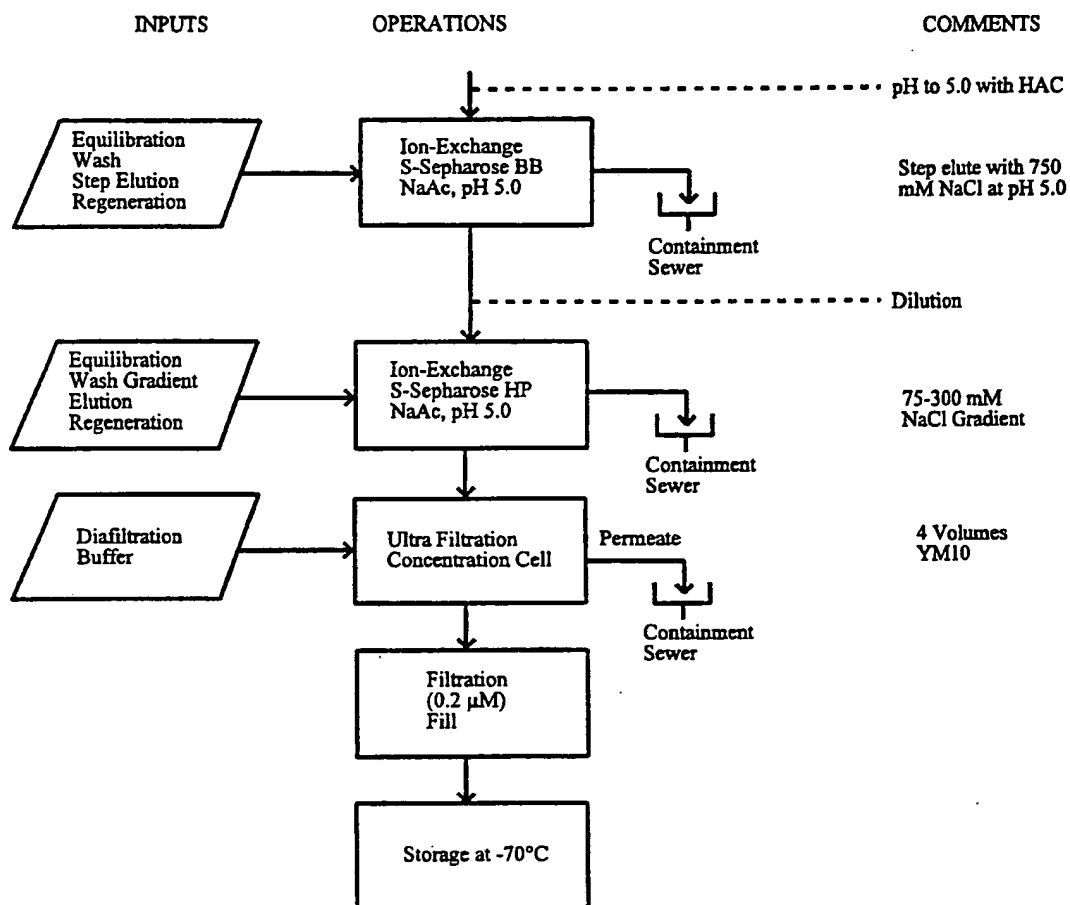


FIGURE 9 (CONTD)



13 / 13

FIGURE 9 (CONTD)



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/05423

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07K 1/14, 14/475, 14/48; C12N 1/20, 15/00, 15/18

US CL :435/69.4, 252.33, 320.1; 530/402, 408, 412, 415, 416, 417

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.4, 252.33, 320.1; 530/402, 408, 412, 415, 416, 417

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and DIALOG (files 5, 155, 351,357, 358) search terms: neurotroph7, coli, refold7, urea, sulfitolysis, sulfonation, PEG, polyethylene glycol, cysteine, disulfide

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 267, No. 19, issued 05 July 1992, J.L. Cleland et al, "Polyethylene glycol enhanced refolding of bovine carbonic anhydrase B", pages 13327-13334, see entire document.	1-26
Y	US, A, 4,620,948 (BUILDER ET AL) 04 November 1986, see entire document.	1-26
Y	EP, A, 450,386 (COLLINS ET AL) 09 September 1992, see entire document.	1-26
Y	US, A, 5,235,043 (COLLINS ET AL) 10 August 1993, see entire document.	1-26

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

25 JULY 1995

Date of mailing of the international search report

03 AUG 1995

 Name and mailing address of the ISA/US
 Commissioner of Patents and Trademarks
 Box PCT
 Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

MARIANNE PORTA ALLEN

Telephone No. (703) 308-0196